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LACTOBACILLUS PLANTARUM MCC2034, A NOVEL ISOLATE FROM TRADITIONAL INDIAN LACTIC FERMENTED PREPARATION: MOLECULAR IDENTIFICATION AND EVALUATION OF ITS *IN VITRO* PROBIOTIC POTENTIAL

David Elisha Henry[1], *Prakash Motiram Halami*[2] *and Siddalingaiya Gurudutt Prapulla**, [1]

Address(es):
[1] Fermentation Technology and Bioengineering department, [2] Food Microbiology department, CSIR-Central Food Technological Research Institute, Mysore – 570020, Karnataka. Phone number: +91-821-2515792.

*Corresponding author: prapullasg@yahoo.co.in

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ABSTRACT

In the present study, the *in vitro* probiotic properties and molecular characterization (16S rRNA gene sequencing) of *L. plantarum* MCC2034, a lactic acid bacterium (LAB) isolated from *Takrarista*, traditionally used for the treatment of various gastro-intestinal maladies are presented. The isolate under study has been proved to be a potent producer of exopolysaccharide (EPS). The molecular identification of the strain was carried out using 16S rRNA gene sequencing method. The ability of the strain to survive the transit through the gastro-intestinal tract (GIT) was carried out using *in vitro* gastric system. Additionally, the *in vitro* adhesion property (*viz.* bacterial adhesion to hydrocarbon assay and autoaggregation) and bile salt hydrolase (BSH) activity of *L. plantarum* MCC2034 were carried out. Antibiotic resistance profile was determined using minimum inhibitory concentration (MIC) test against tetracycline, ampicillin, streptomycin, etc. The results of the *in vitro* probiotic characterization indicated that *L. plantarum* MCC2034 is capable of surviving the adverse conditions encountered during its passage through the GIT besides showing promise with respect to BSH and the antibiotic resistance profile also indicated that the strain is within the recommended limits. In conclusion, it can be stated that *L. plantarum* MCC2034, a *Takrarista* isolate capable of producing EPS, exhibited effective *in vitro* probiotic potential.

Keywords: Exopolysaccharide, LAB, probiotic, fermentation, in vitro, GIT

INTRODUCTION

Ayurveda is an ancient system of medicine that has been practiced in India across millennia. In the last few decades, it has been steadily gaining popularity among the consumers. *Takrarista* is butter milk based lactic fermented preparation containing added spices, which has been traditionally used for the treatment of various gastro-intestinal maladies **(Misra, 1993).** LAB with potent probiotic properties have been isolated and characterized from *Ayurvedic* fermented preparations **(Reddy et al., 2007)**. Additionally, LAB have also been proved to be potent producer of Vitamin B_{12} **(Madhu et al., 2010)**.

Lactic acid bacteria (LAB) have a long history of safe use as microbial agents in fermented food products and have been conferred the ‘‘Generally Recognized As Safe’’ (GRAS) status. Ever since the promotion of LAB as organism capable of imparting health promoting effects by Metchnikoff in the late 19th century, LAB have received wide attention in the field of human health management due to their probiotic nature. The ability to survive the passage through the gastrointestinal tract is essential for a bacterium to be considered as a probiotic strain. Several bacterial species are unable to survive the harsh conditions in the stomach, while others can survive the passage by using their inherent defence mechanisms such as changes in phenotype and gene expression. The adherence ability plays an important role in colonization **(Pedersen and Tannock, 1989; Alander et al., 1999)** and subsequently has been proposed as one of the main selection criteria for potential probiotic strains **(FAO/WHO, 2001)**.

Since the introduction of antibiotics in the twentieth century, emergence of resistant microorganisms has become a major threat to public health. Recently, researchers have also focused on characterizing antibiotic resistance in LAB, which serve as reservoir for antibiotic resistance genes and transfer them to other microorganisms; including pathogens. This situation has become more hazardous by the overuse and misuse of antibiotics as food supplements for humans, or as feed supplements intended for veterinary use. This development has caused a growing interest in the determination of the antibiotic resistance profile, in order to evaluate the safety of the probiotics in their final application as food supplements for humans, or as feed supplements intended for veterinary use **(Nawaz et al., 2011)**.

Before the effective application of the probiotic strains in humans, it is essential that the strain be thoroughly characterized, which brings the taxonomical identification into perspective. The limitations of the phenotypic and biochemical identification methods has provided the impetus for exploring reliable methods for identification of LAB using molecular methods. In this context, 16S ribosomal RNA (rRNA) sequences could be used as evolutionary chronometers. Specific regions of the 16S rRNA molecule are conserved throughout all bacterial species and can be used to align sequences obtained from different isolates. Alignment of these conserved regions permits comparison of the remaining regions which are variable as to nucleotide base sequence between many species. From a practical point of view, the 16S rRNA gene sequences can be used in the reliable identification of many bacterial species through polymerase chain reaction (PCR) based techniques. These molecular approaches allow *Lactobacillus* species to be reliably identified.

EPS are widely produced by LAB. They are long chain polysaccharides made of branched repeating units of sugars (*e.g.*, glucose, galactose, rhamnose, etc.). EPS produced by LAB display a great variety of structures **(De Vuyst and Degeest, 1999)**. EPS of microbial origin are long chain, high-molecular-mass polymers that have potential applications in food industries as texturizers, viscosifiers, emulsifiers and synerisis-lowering agents for their rheological behaviour and water-binding capacity **(Galle et al., 2011)**.

The objective of our study was the molecular characterization and the taxonomical identification of an EPS producing LAB isolated from *Takrarista*, was carried out using the 16S rRNA gene sequencing and the probiotic characterization of the same was evaluated through *in vitro* methods.

MATERIAL AND METHODS

Chemicals

The chemicals used for the present study were of analytical grade, purchased from Sigma-Aldrich Ltd, MO, USA. The microbiological media were purchased from HiMedia Pvt Ltd, Mumbai, India. The primers used for PCR were obtained from Bangalore Genie Pvt. Ltd., Bangalore, India.

Microorganisms and growth conditions

L. plantarum MCC2034 was previously isolated from Takrarista, an indigenous Ayurvedic lactic fermented preparation. *L. plantarum* MCC2034 was maintained as glycerol stocks at -40^{o} C. The strain has been deposited at the Microbial Culture Collection (MCC), at National Cenre for Cell Sciences, Pune, India. The strain was sub-cultured twice in MRS (de Man, Rogosa & Sharpe) broth at 37^{o}C for 24 h prior to use. The strain was selected based on the screening results which indicated its high potential for the production of EPS (unpublished data).

Isolation of chromosomal DNA and amplification of 16S rRNA gene using polymerase chain reaction (PCR)

The total genomic DNA of the isolates was extracted from active cultures grown in MRS broth as described by Mora et al., (2000). The 16S rRNA gene of the targeted LAB strain was amplified by using PCR technique as described by **Khunajakr et al., (2008)**. The PCR product was gel-purified by using Sigma GenElute PCR cleanup kit (Sigma-Aldrich Pvt Ltd., MO, USA), as per the standard protocol. The concentration and purity of the PCR product (3 µl) was confirmed by gel electrophoresis (0.8% agarose gel).

16S rRNA gene sequence analysis

DNA sequencing was carried out using M13F and M13R primers by dideoxy chain termination method **(Sanger et al., 1977)**. The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at Bangalore Genie, Bangalore, India. A sequence based search was performed in GenBank database using BLAST programme (National Centre for Biotechnology Information, Maryland, USA).

Carbohydrate fermentation profile

The carbohydrate fermentation profile of *L. plantarum* MCC2034 was determined by using HiCarbohydrate kit (Himedia Pvt Ltd., Mumbai, India), a standardized colorimetric identification system consisting of thirty five types of carbohydrates (**Table 2**). *L. plantarum* MCC2034 was cultured in MRS broth at 37^{o}C overnight and subsequently washed twice with sterile saline (0.85 % NaCl) and the cells were suspended in equal volume of sterile saline. The cell suspension was inoculated (50 µL) onto each well containing individual carbohydrates under sterile conditions. Subsequent to inoculation, the plates were incubated at 37^{o}C for 18-24 h.

Table 2 Biochemical characterization of *L. plantarum* MCC2034

Carbohydrate Utilization	Reaction
Lactose	+
Xylose	-
Maltose	+
Fructose	+
Dextrose	+
Galactose	+
Raffinose	-
Trehalose	+
Melibiose	+
Sucrose	+
L-Arabinose	-
Mannose	+
Inulin	+
Sodium gluconate	-
Glycerol	-
Salicin	+
Dulcitol	-
Inositol	-
Sorbitol	-
Mannitol	+
Adonitol	-
Arabitol	-
Erythritol	-
α-Methyl-D-glucoside	+
Rhamnose	-
Cellobiose	+
Melezitose	+
α-Methyl-D-mannoside	-
Xylitol	-
ONPG	+
Esculin hydrolysis	+
D-Arabinose	-
Citrate utilization	-
Malonate utilization	-
Sorbose	-

+ indicates utilization and, - indicates non-utilization of the carbohydrate, respectively.

Evaluation of survivability of *L. plantarum* MCC2034 using *in vitro* gastric system

An *in vitro* gastric system model was adopted to simulate the passage through the mouth and stomach **(Mozzi et al., 2008)**. The following components were used: (i) human saliva (6 ml), sterilized at 121^{o}C for 15 min with human α-amylase (Sigma-Aldrich Ltd., MO, USA) at the concentration of 0.76 mg.ml^{-1} (pH: 7.7). The exogenous addition of α-amylase was essential as no enzyme activity was detected after autoclaving the human saliva; (ii) Simulated gastric juice (12 ml), composed of (g/L) KCl, 1.12; NaCl, 2.00; $CaCl_2$, 0.11; KH_2PO_4, 0.40; and mucin, 3.50, adjusted to final pH of 2.0 with HCl and sterilized at 121^{o}C for 15 min. Pepsin (Sigma-Aldrich Ltd, MO,USA), used at 0.1 mg ml^{-1} final concentration, was added prior to experimental use and (iii) sample (9 ml), consisting of *L. plantarum* MCC2034 cultured in MRS and the cells separated and resuspended in saline were incubated with saliva at 37^{o}C for 2 min (final pH 4.6), then gastric juice was added (final pH of 4.25) and the mixture was shaken for 30 min at 37^{o}C to adapt the microorganisms to the acidic conditions of the system. Finally, the mixture was acidified to different pH values in a range of 2.0–3.0 within 2 h by the addition of hydrochloric acid.

Determination of antibiotic resistance profile using minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) of eight antibiotics were determined using the MIC test kit (Himedia Pvt Ltd, Mumbai). The antibiotics evaluated were streptomycin, tetracycline, ampicillin (256-2 µg, 2.048-0.016 µg), chloramphenicol (240-0.01 µg, 8-0.001 µg), vancomycin (256-2 µg, 2.048 – 0.016 µg), erythromycin (240-0.01 µg, 4-0.001 µg), polymixin (240-0.01 µg, 32-0.001 µg) and gentamycin (1024-8 µg, 8.192-0.064).

Bile salt Hydrolase test

The ability of LAB to deconjugate bile salts was determined using the agar plate screening method described by **Du Toit et al., (1998)** and **Franz et al., (2001)** . Overnight cultures were spotted onto MRS agar (Himedia Pvt Ltd., Mumbai) plates containing 0.5% (w/v) sodium salt of taurodeoxycholic acid (Sigma-Aldrich Ltd, MO, USA) and 0.37 g L^{-1} Calcium chloride followed by incubation at 37^{o}C for 24-48 h.

Determination of antagonistic activity

The antimicrobial activity of the strain was evaluated using the soft agar overlay method. The LAB strain, *L. plantarum* MCC2034 was point inoculated on MRS agar medium and incubated at 37^{o}C for 24 h. Subsequently, sterile soft agar (0.7% w/v) was taken in a test tube to which the pathogenic strains were added and mixed thoroughly. The different enteropathogens selected for the experiment were enterotoxigenic *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Yersinia enterocolitica* and *Bacillus coagulans*. The soft agar containing the added pathogenic strains were poured over the MRS agar surface with *L. plantarum* MCC2034 previously inoculated, as detailed above. The plates were incubated at 37^{o}C for 24 h and observed for the development of inhibition zone around the LAB strain.

Surface hydrophobicity test

Surface hydrophobicity of bacteria was determined based on the methods of **Zavaglia et al., (2002)** and **Canzi et al., (2005).** After the growth of *L. plantarum* MCC2034 in MRS broth for 24 h, bacterial cells were harvested by centrifugation at 14,000 g for 5 min, washed twice with sterile saline (0.85% w/v NaCl) and then resuspended in the same to obtain absorbance of about 0.5 at 660 nm (bacterial suspension). Bacterial suspension (5 mL) was mixed with 1 ml of xylene [$C_6H_4(CH_3)_2$] by vortexing for 2 min followed by incubation for 1 h at ambient temperature. The changes in absorbance of bacterial suspension due to bacterial adhesion to hydrocarbons were recorded at 660 nm using spectrophotometer (Shimadzu Corp. Ltd., Japan) Surface hydrophobicity was expressed as surface hydrophobicity percentage (SHb %) and calculated using the formula:

$SHb\ \% = [(A_o - A)/ A_o] \times 100;$

where, A_o and A are the absorbances before and after extraction with xylene.

Production of β-galactosidase by selected LAB

The ability of *L. plantarum* MCC2034 to produce β-galactosidase was carried out as described by **Miller (1972**) with slight modifications.Different carbohydrates such as glucose, lactose and galactose were supplemented to the basal MRS broth to study their influence on β-galactosidase production. Subsequent to the growth of the strain at 37°C for 24 h, the cells were separated by centrifugation (10,000 rpm for 7 min). The cells were washed with 10 mM sodium phosphate buffer (pH 7.0) and suspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 2.7 µL/mL β-mercaptoethanol). The reaction mixture was thoroughly mixed for 2 min followed by incubation at 37°C for 1 h to facilitate the removal of toluene prior to conducting the assay. 200 µL of 200 mM O-nitrophenyl–L-D-galactopyranoside (ONPG, Sigma-Aldrich Ltd, MO, USA) prepared in Z buffer was added and the reaction mixture was incubated at 37°C for 30 min. The reaction was arrested by the addition of 1M Na_2CO_3 (500 µL) and the concentration of O-nitrophenol (ONP) released from ONPG was determined by measuring the absorbance at 420 nm using UV-visible spectrophotometer (UV-1601, Shimadzu Corp., Japan). β-galactosidase activity (Miller units) was calculated using the equation given below:

$$\beta - \text{galactosidase activity} = 100 \text{ x } \frac{15 \text{ min } x \text{ 1 } mL \text{ } x \text{ } A1_{600}}{A_{420} - 1.75 \text{ } x \text{ } A2_{560}}$$

Where, $A1_{600}$ represents the absorbance value prior to the assay; $A2_{560}$ represents the absorbance value of the cell debris and A_{420} represents the absorbance value of the reaction mixture.

RESULTS AND DISCUSSION

Isolation of chromosomal DNA and amplification of 16S rRNA gene using Polymerase chain reaction (PCR)

The molecular characterization of the EPS producing LAB strain, a *Takrarista* isolate using 16S rRNA gene sequencing technique followed by sequence based homology search revealed that the strain is *Lactobacillus plantarum* **(Klijn et al., 1991)** and it was designated as *L. plantarum* MCC2034. The finding is consistent with the earlier reports where strains of *L. plantarum* are found to be frequently associated with naturally fermented preparations that involve materials of plant origin **(Molin, 2001)**. The phenotypic (Gram staining, cell morphology and colony morphology) and the biochemical data (carbohydrate fermentation profile, catalase test, etc.) also concur with the identification of the strain as *L. plantarum* **(Tafti et al., 2013)**.

Carbohydrate fermentation profile

The carbohydrate fermentation profile of *L. plantarum* MCC2034 has been presented in **Table 2**. The carbohydrate utilization pattern was analyzed in comparison with Bergey's manual for the phenotypic characterization of LAB group. The results concur that the strain belongs to LAB group.

Evaluation of survivability of *L. plantarum* MCC2034 using *in vitro* gastric system

An *in vitro* GS model was standardized to evaluate the ability of the strain *L. plantarum* MCC2034 to retain the cell viability. The mixture composed of sample (cells suspended in sterile saline), saliva and gastric juice had a final pH value of 4.25. To approximate the physiological conditions of the stomach after a meal (pH range within 2.0–3.5), the pH of the mixture was decreased until final values of 2.0–3.0 were obtained by adding HCl at different concentrations. The decrease in cell viability for each strain with respect to the initial viability, because of the assayed acidic conditions of the GS model, was statistically significant ($P < 0.05$). The results (**Figure 1)** indicate that the strain is capable of surviving the challenging transit through the gastrointestinal passage before reaching the more habitable colon region, which is the site of colonization for the probiotic strain. The *in vitro* gastric system which was employed for assessing the viability of the strain is intended to provide a better *in vitro* test compared to conventional acid and bile tolerance, since it better simulates the conditions encountered during the GI transit.

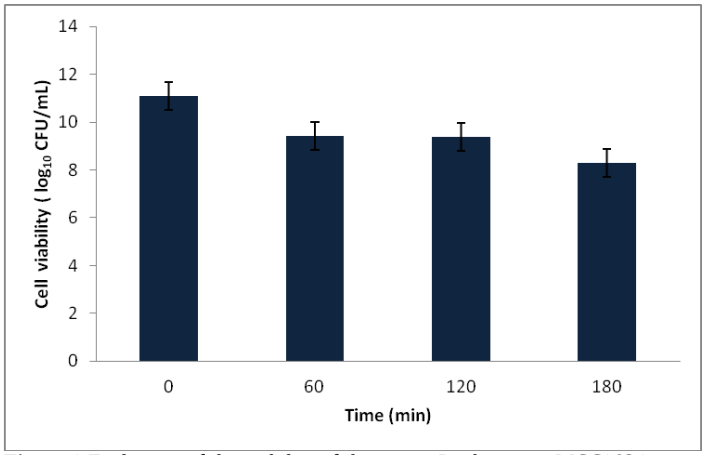

Figure 1 Evaluation of the viability of the strain, *L. plantarum* MCC2034, using *in vitro* gastric system

Determination of antibiotic resistance profile and the MICs of the antibiotics

The MICs of the antibiotics tested are presented in the **Table 1**. From the results, it is evident that the LAB strain, *L. plantarum* MCC2034 exhibited moderate susceptibility to the antibiotics tested. The antibiotics for which the strain *L. plantarum* MCC2034 showed complete resistance across the concentration range towards polymixin and high MIC value towards streptomycin at120 µg/mL. In contrast, the antibiotics for which the strain exhibited susceptibility include erythromycin, chloramphenicol, tetracycline, ampicillin and gentamycin. Interestingly, the MIC value of *L. plantarum* MCC2034 for all the antibiotics tested showed similar values with the exception of gentamycin which showed much higher MIC value. In order for a bacterium to be considered as a probiotic for human application, it is essential that the bacterial strain is resistant to commonly prescribed antibiotics, where it can be administered along with antibiotic therapy to counter its ill-effects. At the same time, it is imperative that the bacterium also be susceptible for certain antibiotics to enable its effective management.

Table 1 Minimum Inhibitory concentration (MIC) values of *L plantarum* MCC2034 against different antibiotics. (Values are represented as mean ± SD)

Antibiotic	MIC value (µg/mL)*
Streptomycin	120 ± 1.154
Tetracycline	0.01± 0.005
Ampicillin	0.01± 0.015
Chloramphenicol	0.01 ± 0.011
Vancomycin	0.01± 0.020
Erythromycin	0.01± 0.011
Polymixin	Resistant
Gentamycin	16 ± 1.527

Bile salt hydrolase test

The LAB strain, *L. plantarum* MCC2034 also tested positive for the bile salt hydrolase plate assay. The ability of a bacterium to deconjugate the bile salt, taurodeoxycholate is considered to be an important attribute of a probiotic since, it causes a beneficial effect in the management of cholesterol levels of the host. The result indicates that the strain has good potential in the management of cholesterol. The physiological concentration of bile acids in the intestine is between 5,000 µM and 20,000 µM **(Hofman, 1991)**. In this study, a concentration of 0.5 % bile salts, equivalent to 12,225 µM was used, which is the range used extensively. As per published reports, it is a notable that *in vitro* studies can only partially mimic the actual *in situ* conditions in the gut ecosystem but nevertheless, provide an important tool in the analysis of specific characteristics prior to carrying out *in vivo* evaluations **(Pinto et al., 2006)**.

Antagonistic acitivity

In the agar spot test, *L. plantarum* MCC2034 displayed moderate to strong antagonism towards test strains *viz. S. aureus*, Enterotoxigenic *E. coli*, *L.monocytogenes*, *S. enteritidis and Y. enterocolitica*. The inhibition zones formed around the *L. plantarum* MCC2034 colony ranged from 3-10 mm in radius. The gut epithelium provides a challenging environment for any microorganism and the successful colonization of the same depends on the capability of the microorganism to compete and establish itself against the local microflora. For a probiotic bacterium, the ability to display antagonistic activity against other microorganisms is crucial not only for its successful colonization of the gut epithelia but also an effective way of inhibiting the colonization of pathogenic microorganisms, thereby providing an effective barrier **(Hudault et al., 1997)**.

Surface hydrophobicity test

The results of the BATH (Bacterial Adhesion To Hydrocarbons) test indicated that the LAB strain, *L. plantarum* MCC2034, exhibited moderate adhesion with an H% (hydrophobicity) of 30.38%. *In vitro* hydrophobicity tests are used as an indicator of the ability of the test strain to adhere to the intestinal epithelium, since the cells that comprise the intestinal epithelium display hydrophobic surface properties **(Perez et al., 1998; Pan et al., 2006)**.

Production of β-galactosidase by selected LAB

Lactose intolerance is a metabolic condition characterized by the inability to digest lactose which leads to discomfort upon the consumption of milk products. This condition results from the insufficient amount of β-galactosidase production in the intestine and due to the condition, lactose intolerant people prefer to avoid milk in their diet **(Cebeci and Goukan, 2003)**. LAB are widely acknowledged for the production of β-galactosidase, which helps in the breakdown of lactose before it reaches the colon, where it is subjected to rapid degradation by the colon microflora. This enzyme can help in the preparation of low-lactose milk products and biosynthesis of galactooligosaccharides that are interesting from the technological as well as health point of view. In the present study, the β-galactosidase assay for *L. plantarum* MCC2034 indicated that it is a potent producer of β-galactosidase with an activity of 2300 MU (Miller units).

CONCLUSION

In conclusion, it can be stated that the EPS producing LAB isolated from *Takrarista*, was identified using 16S gene sequencing method as *Lactobacillus plantarum* and designated the unique strain number MCC2034. The studies on the *in vitro* probiotic properties of *L. plantarum* MCC2034 indicated that the strain is a potent probiotic strain capable of surviving passage through the GIT, and also possessing an assortment of desirable characteristics that provide added functionality for its application as an EPS producing probiotic LAB. The results of the present work also warrant further studies using *in vivo* model to substantiate the positive results obtained in the *in vitro* model.

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